Mechanistic insight to mycoremediation potential of a metal resistant fungal strain for removal of hazardous metals from multimetal pesticide matrix

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ABSTRACT

Fungi have an exceptional capability to flourish in presence of heavy metals and pesticide. However, the cue for mycoremediation of pesticide (lindane) and multimetal (mixture of cadmium, chromium, copper, nickel, lead, zinc) by a fungus is little understood. In the present study, *Aspergillus fumigatus,* a filamentous fungus was found to accumulate heavy metals in the order [Zn(98%)>Pb(95%)>Cd(63%)>Cr(62%)>Ni(46%)>Cu(37%)] from a cocktail of 30 mg/L multimetal and lindane (30 mg/L) in a composite media amended with 1% glucose. Particularly, Pb and Zn uptake was enhanced in presence of lindane. Remarkably, lindane was degraded to 1.92 ± 0.01 mg/L in 72 h which is below the permissible limit value (2.0 mg/L) for the discharge of lindane into the aquatic bodies as prescribed by European Community legislation. The utilization of lindane as a cometabolite from the complex environment was evident by the phenomenal growth of the fungal pellet biomass (5.89 ± 0.03 g/L) at 72 h with cube root growth constant of fungus (0.0211 $g^{1/3}$ $l^{-1/3}$ h^{-1}) compared to the biomasses obtained in case of the biotic control as well as in presence of multimetal complex without lindane. The different analytical techniques revealed the various

stress coping strategies adopted by *A. fumigatus* for multimetal uptake in the simultaneous presence of multimetal and pesticide. TEM coupled with energy dispersive X-ray analysis results deduced uptake of Cd, Cu and Pb in the cytoplasmic membrane while the metals Cr, Ni and Zn accumulated in the cytoplasm of the fungus. Fourier-transform infrared spectroscopy (FTIR) revealed involvement of carboxyl/amide group of fungal cell wall in metal chelation. Thus *A.fumigatus* exhibited biosorption and bioaccumulation as the mechanisms involved in detoxification of multimetals.

Capsule: *A.fumigatus* PD-18 implements exclusive and variable strategies viz. extracellular and intracellular complexation of metals and lindane from a multimetal and lindane matrix.

Keywords: multimetal; lindane; TEM-EDAX; FTIR; bioaccumulation; biosorption

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1. Introduction

Decontamination of pollutants such as heavy metals and pesticide is a pressing need in the developing countries like India. The Central Pollution Control Board (CPCB), India and Food and Agriculture Organization (FAO) maintain a list of hazardous pollutants (including cadmium, total chromium, copper, nickel, lead and zinc) which are frequently released into the environment such as surface water (lake, river, stream etc.) through discharge from various small-scale industries like preservatives, fertilizer, pesticide, electroplating, batteries, textile etc. These small and medium-scale enterprises do not comply with strict guidelines for effluent treatment as they are unable to invest in expensive pollution control treatment equipment and ceaselessly discharge the effluents containing heavy metals into the major rivers like Yamuna and other water bodies (Bhattacharya et al., 2015).

In addition to the cocktail of metals, residues of organochlorine pesticides like gamma-hexachlorocyclohexane Lindane and DDT and organophosphorus pesticides like Malathion and Chlorpyrifos persist in the river water like Yamuna (Mutiyar et al., 2011; Pandey et al., 2011).These chemicals are banned by Central Insecticides Board and Registration Committee (CIBRC), India but are rampantly used in agriculture to control weeds, pests and diseases in crops as these are cheaper and lucrative options for the farmers (Walker et al., 1999).This leads to the seepage of these chemicals into the water bodies such as groundwater through agricultural runoffs.This impacts the agricultural lands by way of variable loss at carbon and nitrogen content in the top soil measuring about 20 cm (Fromin et al.,2012).The crops especially irrigated with contaminated water become the carriers of heavy metals and pesticide (Mandal and Singh, 2010;Singh et al., 2010).

These agrochemicals then enter in our food chain and pose a major cause of health implications such as cancer, neurological diseases etc. as enlisted by Agency for Toxic Substances and Disease Registry (ATSDR) and US Environmental Protection Agency (EPA).

Therefore, it becomes pertinent to eliminate these hazardous pollutants from these natural resources *ex-situ*.

Plants (Müller et al.,2013;Yadav et al., 2010) and microorganisms such as algae (Chaurasia et al., 2013; Davis et al., 2003; González et al., 2012), bacteria (Amoozegar et al., 2007; Lee et al., 2019), fungi (De Lima et al., 2013; Mishra and Malik, 2012; Sagar and Singh, 2011) and yeast (De Silóniz et al., 2002) as well as microbes in activated sludge (Atkinson et al., 1998; Zheng et al.,2018) have played key roles in the remediation of heavy-metals and pesticide-contaminated soil and water. Hence extensive research and development have occurred on biological methods for remediation of heavy metal and pesticide contamination (Mishra and Malik, 2013; Shah, 2016).

However, only few of these studies have utilized growing microbial cells which develop multiple tolerance mechanisms for the combined metal/pesticide through production of enzymes and thus holds greater metal/pesticide uptake capacities (Malik, 2004; Mishra and Malik, 2013). Also, it is difficult to introduce genetically modified organisms into the natural system due to regulatory constraints. Thus, indigenous microorganisms able to remediate multimetal and pesticide may be more desirable.

Additionally, utilizing a well acclimatized filamentous fungus has a clear advantage for bioremediation applications over single celled organisms as it provides a richer metabolic network and have improved capacity to adept to environmental limits like extremes of temperature, pH, high metal and pesticides concentrations and limiting nutrient availability due to their morphological diversity. The growth of fungal mycelia maximizes both the mechanical and enzymatic contact with the pollutant due to increased cell to surface ratio (Sagar and Singh, 2011). Particularly fungi from the genus *Aspergillus* are noted for remediation of vast array of noxious compounds ranging from textile dyes, heavy metals, pesticides, aromatic compounds etc.(Deshmukh et al., 2016).Besides, fungi tend to produce a

suite of extracellular enzymes, exopolysaccharides, proteins, organic acids and other metabolites that assist in metal/pesticide remediation (Kaushik and Malik, 2011).

1.1. Rationale of this study

The individual metal removal capacity of a microorganism is an intricate process that depends on the cell composition and physiology of the microorganism. It also depends the on factors viz. composition and concentration of the metals and on the chemistry of the metal ions (Gadd, 2007).Also, the individual metals have a distinctive interaction among themselves when present in a complex multiple co-contaminant system which could be synergistic, antagonistic, or noninteractive and compete for the metal binding sites in the microorganisms (Pakshirajan and Swaminathan, 2009). Further, hydrophobic and lipophilic compounds, e.g. pesticides, interact with the organic matrix on the biomass surface and membranes which results in adsorption of the organic compounds (Gadd, 2008).Limited studies have been conducted for the bioremediation of single metal and single pesticide mixture (Thakur and Srivastava, 2011) or ternary metals (Kumar et al., 2016) or binary pesticide mixture (Liang et al., 2014).

Gola et al. (2019) evaluated the uptake of the individual metals from a cocktail of hexametals (Cd, Cr, Cu, Ni, Pb and Zn) mixture by the fungus *Beauveria bassiana*. Their study revealed the preferential uptake of the individual metals (99.6% Pb, 99.9% Cr, 85.6% Cu, 57.4% Zn, 62.4% Ni and 95% Cd) by the fungus and a removal of 83.3% of the total hexametals when present in solution.

Additionally, in a system of organic compound and heavy metals, the effect of metals on the degradation of the organic compound is complex which is depends on the physico-chemical and biological characteristics of the contaminated systems (Moreira et al., 2013). Therefore, it is essential to assess the bioremediation of pollutants in a mixture to facilitate the selection of a robust strain for bioremediation.

Aparicio et al., (2018) investigated the performances of single and mixed cultures of actinobacteria (the actinobacteria *Streptomyces* sp. M7, MC1, A5 and *Amycolatopsis tucumanensis* DSM 45259) for the remediation of pesticide lindane and heavy metal chromium from a co-contaminated environment. The highest removal of chromium and lindane from simulated soil sample were 50% and 60%, respectively. While the removal potential of the quadruple consortium for the same contaminants were 60% and 55%, respectively. This reflected the synergism amongst the microbial consortium for combining their metabolic activities for the selective removal of the heavy metal chromium but not for the pesticide lindane. Thus, investigations involving mechanism and methodologies for simultaneous remediation of organic and multiple inorganic contaminants needs to be carried out.

1.2. Our contribution

This study uniquely details the mechanistic insight of biosorption and bioaccumulation of hexametals into the *Aspergillus fumigatus* biomass and the degradation process of pesticide lindane from a simulated hexametals and lindane pesticide matrix which reflect the actual co-contaminated water systems. Hence this understanding would aid in the development of a better, evolved and cost-effective remediation technique which can be disseminated for bioremediation of river water *ex-situ* for various agronomical purpose.

Materials and methods

2.1. Chemicals and reagents

Different individual metal stock solutions of 10 g/L were prepared by dissolving their respective salts $Cd(NO_3)_2$, $K_2Cr_2O_7$, $Cu(NO_3)_2$, $Ni(NO_3)_2$, $Pb(CH_3COO)_2$ and $Zn(NO_3)_2$ in double distilled water and was diluted to obtain the required concentrations prior to use in the experiments. Lindane stock solution of 100 g/L was prepared by dissolving the lindane powder in HPLC grade acetone. To prepare the reagents and calibration standards, deionized

144 ultrapure water (RIONS Ultra 370 series) was used. All the other chemicals used were of
145 analytical grade and were obtained from Merck, Sigma and Qualigens.

146 **2.2. Microorganism and culture media composition**

147 The fungal strain was previously isolated from the soil sample collected from the Okhla
148 sampling site of the Yamuna river, New Delhi, India and characterized as *Aspergillus*
149 *fumigatus* PD-18 with accession number KX365202 after submitting the sequence to the
150 Genbank (NCBI) (Dey et al., 2016).

151 The growth media used was composite medium and the composition of the media was as
152 follows (g/L): (K_2HPO_4, 0.5; NH_4NO_3, 0.5; NaCl, 1.0; $MgSO_4.7H_2O$, 0.1; Yeast extract, 2.5;
153 pH 6.8 ± 0.2). The media was sterilized at 121˚C for 15 minutes. Glucose 10.0 g/L was
154 autoclaved separately and then added to the flasks to avoid precipitation.

155 **2.3. Multimetal bioaccumulation, lindane degradation and growth kinetics of the fungal**
156 **strain in the presence and absence of lindane**

157 The approach for this study was to first evaluate the *A.fumigatus's* metal accumulation in the
158 presence of multimetal and thereafter study metal sequestration under the exposures of
159 multimetal and pesticide stress in composite broth. The effect on growth, substrate (glucose)
160 consumption, multimetal removal as well as lindane degradation was studied at fixed time
161 intervals for a period of 72 h.

162 Firstly, the metal bioaccumulation potential and growth kinetic of *A.fumigatus* PD-18 was
163 studied in presence of 30 mg/L multimetal (5 mg/L of each of the individual Cd, Total Cr,
164 Cu, Ni, Pb and Zn), respectively, in addition to 1% of glucose in the composite media. This
165 was carried out keeping in view of the permissible limits for the presence of each metal in
166 irrigation water according to Food and Agriculture Organisation (FAO) (Cd,0.01;Total Cr,
167 0.1;Cu,0.2; Ni,0.2; Pb,5.0; Zn,2.0) as well as the occurrence of the metals in the river body
168 (Bhattacharya et al., 2015).Secondly, metal bioaccumulation, growth kinetic and lindane

169 degradation potential of *A.fumigatus* PD-18 was studied by amending the composite media
170 with 30 mg/L multimetal, 30 mg/L lindane in addition to 1% of glucose. The biotic control
171 comprised of composite media amended with only 1% of glucose. Two abiotic uninoculated
172 controls comprised of media with only 30 mg/L multimetal as well as media with 30 mg/L
173 multimetal + 30 mg/L lindane.
174 Studies were performed in series of Erlenmeyer flasks (250 mL) containing 100 ml of
175 composite growth media. The flasks were inoculated with 1ml of spore suspension (having a
176 concentration of 10^7 spores eluted with sterile distilled water containing 0.01% Tween 80)
177 and incubated at a temperature of 30˚C and agitated at 150 rpm constant shaking rate for 72 h
178 to ascertain complete accumulation of metal ions. At every 6-hour interval, flasks were
179 withdrawn and the dry weight of the resultant biomass was measured gravimetrically. The
180 pellet mass was estimated after filtration of the contents on pre-dried and pre-weighed
181 Whatman No.1 filter paper and drying it overnight at 60˚C in oven. Also, the supernatant
182 from each flask was analysed for residual glucose, residual metal ion concentration, residual
183 lindane concentration in the solution after centrifugation at 5000 rpm for 10 min. All the
184 analytical measurements were done in triplicate. The amount of metals accumulated by the
185 fungal strain was calculated from the difference between the initial concentration and the
186 concentration remaining in the supernatant. The experimental data obtained from the batch
187 multimetal, accumulation, lindane degradation and fungal growth experiments were subjected
188 to cube root equation
189 $M^{1/3} = kt + M_0^{1/3}$ (Papagianni, 2004).
190 Where M =dry cell weight (g/L) of the fungal cells at time t (h), M_0= initial dry cell weight
191 (g/L), k = cube root kinetic constant ($g^{\mathbf{1/3}}l^{-\mathbf{1/3}}h^{-1}$).
192 Specific uptake capacity, qm (mg/g) of the fungal biomass was determined using following
193 equation

194 (q_m) = total metal removed (mg/L)/total dried biomass (g/L)

195 **2.4. Analysis of residual heavy metals and estimation of residual sugar content**

196 Metal ions (cadmium, chromium, copper, nickel, lead and zinc) concentration in water
197 sample were determined after digestion with concentrated nitric acid (65%) in the ratio 1:10
198 (nitric acid: sample) in microwave digester (Anton Paar Multiwave Pro) using standard
199 method (Eaton and Franson, 2005).The digested solution was quantified through Atomic
200 Absorption Spectroscopy (ECIL AAS4141).Residual sugar content in the samples were
201 analysed by the reagent dinitrosalicyclic acid method (DNS) for reducing sugar (Miller,
202 1959) at 540 nm using spectrophometer (Perkin Elmer Lamda 35 UV/Vis systems).

203 **2.5. Estimation of residual lindane concentration**

204 Residual lindane in the samples were estimated by adding the sample solution to ethyl
205 acetate. This led to formation of two layers of organic and aqueous phase. The organic phase
206 (ethyl acetate) was transferred to another falcon tube which was evaporated by rotary
207 evaporator. Left behind residual lindane in the falcon tube was diluted by HPLC grade ethyl
208 acetate and estimated by Gas Chromatography (Agilent 7890A). The oven temperature was
209 programmed to 180˚C while the injector and interface temperatures were kept at 220˚C and
210 250˚C, respectively. The carrier gas used was nitrogen. Lindane degradation rate constant (k)
211 was determined by plotting the lindane concentrations at different time intervals versus time
212 on semi logarithmic graph.

213 $\ln C = -Kt + \ln C_0$

214 Where C is the lindane concentration at 72 h and C_0 is the initial lindane concentration

215 **2.6. Study of mechanism of metal bioaccumulation and biosorption by the analytical**
216 **instruments at physiological level**

217 The study was done to understand the metal uptake mechanism under multimetal as well as
218 multimetal plus pesticide exposure. The selected fungal strain *A.fumigatus* PD-18 was grown

in composite media and incubated till the fungus growth reached the late log phase (120 h) and the pellets were harvested thereafter. The morphological changes due to these stresses as well as the localisation of the multimetals inside the fungal biomass were assessed utilising the tools such as Scanning Electron Microscope (SEM); Fourier Transform Infrared Spectroscopy (FTIR); Transmission Electron Microscopy coupled with EDX.

2.6.1. Scanning Electron Microscopy (SEM)

The fungal pellets were harvested from the flasks after 120 h of incubation period and were subsequently washed with phosphate buffer (pH 7.2). This treatment was followed by fixation of the fungal pellets in 2.5% glutaraldehyde for 12-18 h at 4˚C.The pellets were then dehydrated by washing with different series of dilutions of ethanol (30%, 50%, 70%, 90% and absolute alcohol). Afterwards the fungal specimens were gold-coated by cathodic spraying (Polaron gold) and examined under a scanning electron microscope (ZEISS EVO 50) at an acceleration voltage of 20 kV with magnification: = 5000 X.

2.6.2. Fourier Transform Infrared spectroscopy (FTIR)

The fungal biomass was harvested after 120 h from the media and were first washed with phosphate buffer (pH 7.2) followed by air drying and lyophilisation. The FTIR spectra for the dried fungal pellets were recorded using Spectrum One (Perkin Elmer) spectrometer equipped with DTGS detector.IR absorbance data were obtained for the wave number range of 520-4000 cm^{-1}.The interpretation of the FTIR spectra were compared with standard spectra of various organic compounds (Silverstein et al., 2005).

2.6.3. Transmission Electron microscopy coupled with Energy Dispersive X-Ray spectroscopy (TEM-EDX)

The fungal biomass was harvested after incubating for 120 h and then washed with phosphate buffer (pH 7.2). The pellets were subsequently fixed in 1% glutaraldehyde and 2% paraformaldehyde for 12-18 h at 4 □ C. The cells were subsequently washed in fresh buffer

and then fixed again for 2 h in osmium tetra oxide (1%) in phosphate buffer at 4˚C. After washing in buffer, the specimens were dehydrated in graded acetone solution followed by embedding the specimens in CY 212 araldite. The specimens were then cut into ultrathin section of 60-80 nm thickness using an ultramicrotome (Leica EM UC 6) followed by staining in alcoholic uranyl acetate for 10 min and lead citrate for 10 min. The grids were then examined in transmission electron microscopy (JEOL JEM-2100F) operated at 120 kV.

2.7. Statistical analysis of the data

All the experiments were conducted in triplicates. Statistical calculations were performed with SPSS 21. Significant effect of lindane concentration on metal uptake was calculated by means of one-way multivariate analysis of variance (MANOVA) using Wilk's lambda test statistic. The dependent variables (metals) were tested for homogeneity of variance using Levene's test.

3.Results

The results obtained in this study depicted the notable growth of *A.fumigatus* PD-18 in presence of multimetal and pesticide lindane. Furthermore, the tools such as SEM, TEM-EDAX mapping and FTIR revealed the biosorption and bioaccumulation of metals in the fungal biomass.

3.1. Growth, glucose utilization, metal accumulation and pesticide degradation potential of *A.fumigatus*

A.fumigatus was able to utilize glucose under multimetal (MM) as well as multimetal+pesticide exposures (MML) and glucose was completely consumed in ~72 h **(Fig.1)**.Further, glucose was consumed in 30 h and 60 h for biotic control (BC) and under multimetal stress, respectively **Supplementary Fig. S1**.

This strain also aided in effective degradation of lindane. It is apparent that rapid removal of lindane (62% of the initial concentration) was observed in first 6 h. The residual lindane

concentration decrement was in parallel to the fungal growth, achieving 96% lindane removal at the end of 72 h **(Fig.1)**.

The result of gas chromatograph (GC) is also shown **Supplementary Fig.S4 (a-b).** The chromatogram shows the residual concentration of lindane in the medium taken at 0 h and after 72 h growth period. The GC analysis revealed a single peak of lindane with retention time ~8.7 minutes.

Lindane concentration in the abiotic non-inoculated control setup remained constant implying no abiotic loss. A first-order kinetics model was used to determine the rate constants (k) for lindane degradation in the presence of co-substrate glucose. The degradation rate constant (k) was found to be 0.0289 h^{-1}.

The fungal pellet growth during the degradation of lindane in the presence of glucose and multimetal is shown in **Supplementary Fig. S2**.Maximum biomass production by ***A.fumigatus*** in the three types of exposures viz. multimetal and pesticide free biotic control (BC), 30 mg/L multimetal (MM) and 30 mg/L multimetal + 30 mg/L lindane (MML) were as follows MML (5.89 ± 0.03 g/L) at 72 h > BC (5.42 ± 0.01 g/L) at 36 h > MM (4.59 ± 0.01 g/L) at 66 h **(Table.1)**.

The comparable cube root growth kinetic constant k of the fungal strain evaluated in case of three types of exposures were as follows BC (0.0562 $g^{1/3}L^{-1/3}h^{-1}$) > MM (0.0226 $g^{1/3}L^{-1/3}h^{-1}$) > MML (0.0211 $g^{1/3}L^{-1/3}h^{-1}$) for the strain *A.fumigatus* **(Table.1)**.

In **Supplementary Fig. S3**, 5% of multimetal had been removed from medium in the initial 6 h in the presence of lindane. After 6 h, multimetal removal almost gradually paralleled the lindane degradation **(Fig.1)**. However, the metal accumulation potential of the strain was affected in the presence of lindane. After 72 h of cultivation, the final concentration of residual multimetal concentration in the medium in case of both the exposures were (MML) 9.92 ± 0.001 mg/L and (MM) 2.41 ± 0.001 mg/L, respectively **Supplementary Fig. S3**. The

294 multimetal concentration in both the abiotic non-inoculated controls ie. media containing
295 multimetal and media containing multimetal + lindane remained same. Correspondingly the
296 total multimetal accumulation by *A.fumigatus* were calculated as (MML) 20.04 ± 0.02 mg/L
297 and (MM) 27.59 ± 0.09 mg/L, respectively **(Table.1)**.
298 Among all six metals, the removal percentages **Fig.2**, based on metal accumulated from the
299 medium in 72 h, declined in the presence of lindane in the order of
300 Zn (98%)>Pb (95%)>Cd (63%)>Total Cr (62%)>Ni (46%)>Cu (37%).
301 Overall there was significant accumulation of metals in the absence of lindane. The metal
302 removal trend was Cd (100%)>Ni (99%)>Cu (98%)>Pb (91%)>Zn (84%)>Cr (81%).
303 The addition of lindane had significant effect on the accumulation of all the metals viz. Cd,
304 Cr, Cu, Ni, Pb as well as Zn. (Here $p<0.05$ according to one-way MANOVA) as shown in
305 **(Table.2).** The Wilks' Lambda has a p-value <0.0001.

306 **3.2. Physiological analysis of metal accumulation by *A.fumigatus***

307 **3.2.1.SEM analysis**

308 The SEM micrographs showed a clear distinction between the control **Fig.3 (a)** and the
309 mycelia stressed with 30 mg/L multimetal and 30 mg/L multimetal + 30 mg/L lindane **Fig.3**
310 **(a-c)**. The fungal hyphae in control is loosely packed and ribbon like in multimetal free and
311 lindane free biomass **Fig.3 (a).** The mycelia in the presence of 30 mg/L multimetal brought
312 slight shrinkage **Fig.3 (b)**. In case of treatment with 30 mg/L multimetal + 30 mg/L lindane,
313 the fungal biomass exhibited rough surface.

314 **3.2.2. TEM-EDX analysis**

315 The TEM-EDX micrographs as shown in **Fig.4 (a) (I-IX),** revealed that the fungal isolate
316 accumulated Ni and Zn in its cytoplasm while Cd, Cr, Cu and Pb in cell membrane/cell wall
317 in case of 30 mg/L initial multimetal. When the fungal biomass was exposed to 30 mg/L
318 multimetal+30 mg/L lindane, this strain had tendency to accumulate more of Cr in the

cytoplasm besides the metals Ni and Zn. While in the cell membrane/cell wall there was accumulation of metals Cd, Cu, and Pb as depicted in the figures **Fig.4 (b) (I-IX)**.

3.2.3. FTIR analysis

FTIR spectra for fungal biomass grown in absence of metal (biotic control), multimetal laden as well as in the presence of pesticide **(Fig.5)** exhibited the various functional groups involved in the multimetal binding in infra-red spectra range (520-4000).The dominant functional groups present in biotic control are O-H and N-H stretching (3000-3500 cm^{-1}), C-H stretching (2800-3000 cm^{-1}) C=O stretching in carboxyl or amide I band groups (1600-1700 cm^{-1}),N-H bending in amide III and C-N stretching in –CO-NH- groups (1500-1600 cm^{-1}), C-OH and C-O-P stretching (1000-1200 cm^{-1}), nitro and disulphide groups (800-850 cm^{-1}). In case of the 30 mg/L multimetal laden biomass there were masking of the C-H stretching group which were evident from the disappearance of the peaks as compared to the control biomass. There was masking of OH and NH stretching groups which were evidently involved in multimetal as well as in multimetal + pesticide treated samples. Also, there was disappearance of the carboxyl/amide group stretching and thiol group stretching as observed in case of 30 mg/L multimetal and 30 mg/L lindane laden biomass.

4.Discussion

4.1. Growth, glucose utilization, metal accumulation and pesticide degradation potential of *A.fumigatus*

The findings of this study clearly shows that *A.fumigatus* could tolerate and degrade lindane by cometabolism with amendment of 1% glucose which served as an energy source and aided in rapid lindane degradation and consequently in higher biomass yield of *A.fumigatus* **(Fig.1)**. It is important to note that *A.fumigatus* could not grow with only lindane as a sole carbon source in composite medium in 72 h (data not shown).

343 It is known that the breakdown of organic compounds is associated to an inducible system.
344 Where occurrence of heavy metals in a co-contaminated system may impede the degradation
345 of the organic pollutants by competing with essential metal cofactors and enzymes eg.
346 oxygenases and consequently reduce the enzymatic activity in microorganisms (Sandrin and
347 Maier, 2003).On the contrary, this fungal strain *A.fumigatus* on addition of 1% glucose was
348 able to degrade 30 mg/L lindane to 1.92 ± 0.01 mg/L in 72 h which is below the permissible
349 limit value (2.0 mg/L) for the discharge of lindane into the aquatic environment according to
350 European Community legislation **(Fig.1)**.
351 Organic compounds contamination may derail the primary substrate (glucose) utilization by
352 microorganisms. Correspondingly, in this study there was interference of glucose utilization
353 and glucose consumption was delayed with the introduction of pesticide simultaneously in
354 the presence of multimetal. It was observed *A.fumigatus* could use glucose only after the first
355 48 h of incubation, resulting in sequential 15% glucose removal and 83% lindane degradation
356 **Supplementary Fig. S1**.
357 Other researchers too have enumerated the direct correlation between the microbial growth
358 and its degradation ability along with cometabolism. De Schrijver and De Mot,(1999) studied
359 cometabolism in the presence of a carbon source (glucose) in the actinomycetes. They
360 concluded that pesticides serve as carbon, nitrogen or phosphorous source through partial
361 transformation reactions. The simple growth substrates (carbon sources viz. fructose, glucose
362 or small chain fatty acids viz. sodium succinate or sodium acetate) promote cell growth,
363 enhance the degradation of heavy molecular weight organic substrate (pesticide) and also act
364 as a cosubstrate and aid cometabolism by inducing production of catabolic enzymes such as
365 laccase, peroxidase (Shan et al., 2009) or heme thiolate monooxygenases (Mougin et al.,
366 1997).

The biodegradation of lindane and the complete utilization of glucose is evident from the growth of the *A.fumigatus*. Though lindane derailed the growth of fungal biomass, which required an acclimatization phase for growth to occur **Supplementary Fig. S2**. The lag phase extended to ~18 h multimetal (MM) and ~36 h multimetal + lindane (MML), respectively, as the complexity of contamination increased. Thus the current growth profile clearly indicates the adaption of *A.fumigatus* to MM and MML exposure during extended lag phase.

Remarkably, in this study, while there was slow growth under MM exposure, MML exposure resulted in higher growth beyond 48 h and the biomass obtained was more than the biomass obtained in the biotic control **Supplementary Fig. S2**. This observation evidently indicates the degradation of the organic contaminant lindane as well as the utilization of its breakdown products as carbon source for the growth of the fungus. Additionally, with the introduction of organic contaminant (pesticide), the overall growth rate of the fungus decreased. The higher biomass obtained in case of MML may be due to the delayed lag phase resulting in the slow utilization of the lindane degradation products **(Table 1)**.

Also, the extent of inhibition of metal uptake by the fungal strain *A.fumigatus* can be postulated with the metabolism of the lindane due to the formation of metabolites as evident from the appearance of new peaks in chromatogram beyond 6 h **Supplementary Fig. S4 (b)**.Other researchers too noted the formation of metabolites. Guillén-jiménez et al.,(2012) and (Mougin et al., 1996) also reported the formation of metabolites like benzoic acid, penta chlorocyclohexane or tetra chlorocyclohexene, tetra chlorocyclohexene epoxide, tetra chlorocyclohexenol by the degradation of lindane by fungi. Thus, fungus breaks down the recalcitrant lindane into its metabolites that alleviates the suppression of lindane biodegradation which could be brought about by the presence of multimetals (Cd, Cr, Cu, Pb and Zn).

391 The multimetal uptake by *A.fumigatus* was initiated after few hours of incubation in case of
392 lindane amended medium, **Supplementary Fig. S3**. It is evident that the multimetal uptake
393 was less and disrupted in the presence of lindane. This indicated an extended lag phase,
394 followed by slow utilization of the primary growth substrate glucose which led to less
395 biomass formation and consequently slow and less metal uptake.
396 The metal removal trend in the absence of lindane was Cd (100%)>Ni (99%)>Cu (98%)>Pb
397 (91%)>Zn (84%)>Cr (81%) (**Fig.2)**. Cd was completely removed while the metals Ni, Cu
398 and Pb had almost similar percentage of removal. This selective metal removal efficacy in a
399 multiple metal system depends on metal ion characteristics such as electronegativity of the
400 individual ions (Açikel and Alp, 2009).The fungal biomass surface exhibits a negative charge
401 due to the inorganic and organic groups being ionized. The most electronegative metal ion
402 will be more strongly attracted to the surface of the biomass. The electronegativity of the
403 metal ions follows the order Zn≤Cr<Cd<Cu≤Ni<Pb. Therefore, in this multimetal mixture
404 environment, bioaccumulation of Zn and Cr decreased consistently as they are less
405 electronegative and would have less affinity towards the fungal biomass. Nonetheless, the
406 metals Cd, Cu, Ni, Pb and Zn were brought down below the permissible mandates of FAO.
407 The percentage of metal accumulated from the multimetal + lindane amended medium in 72
408 h **(Fig.2)** was in the order of Zn (98%)> Pb (95%)> Cd (63%)> Total Cr (62%)> Ni (46%)>
409 Cu (37%). This decreased uptake of metals by the fungus in the presence of a co-contaminant
410 is attributed to the phenomenon of screening effect by the pesticide lindane. Presence of
411 lindane that is adsorbed as shown in the SEM micrograph **Fig.3 (c)** can cover the sorption
412 sites on cell surface and act as a blanket. This was further established by the FTIR results
413 **(Fig.5)** that depicted low molecular weight organic acids viz. carboxylic and amino groups of
414 the fungal cell membrane (Liu et al.,2017) which were found to be binding with the pesticide
415 lindane. Thus, these factors resulted in the reduced binding of the metals.

On the basis of the metabolism of the fungus, it can be hypothesized that in the presence of lindane, the slow uptake of metals could be attributed to the energy provided by the electron acceptors NAD^+. The degradation of lindane and the accumulation of metals may not take place simultaneously. At first instance energy in the fungus maybe channelized for the NADH dependent lindane breakdown. On the second instance, the residual energy after lindane degradation may be utilised for the uptake of more soluble and bioavailable metals viz. Cd, Cu, Ni and Zn. Metals like Cr and Pb are less soluble (Igiri et al.,2018). However, it can be postulated that Pb having higher atomic number and a soft acid has the affinity to bind with the thiol group (ligand) in the metallothionein occurring in the cell membrane of the fungus (Gutiérrez et al.,2019, Nies,1999) as depicted in the TEM-EDAX micrograph **Fig.4 (b) VII.**

Interestingly, *A.fumigatus* exhibited higher removal of Pb (95%) and Zn (98%) in the presence of lindane than in the absence of lindane **Fig.2**.This phenomenon of elevated Pb and Zn sequestration in the fungal cell could be attributed to the enhanced permeability of the cell wall due to the change in the cell structure dynamics by the extreme toxicity of the pesticide (Słaba et al.,2009).The researchers observed that increase in zinc binding by the growing mycelium of *P.marquandii* in the presence of the pesticide was caused by the changes in the wall and membrane composition induced by simultaneous toxic interaction of zinc and alachlor. Another study revealed that polyaromatic hydrocarbons may damage microbes and network with the lipophilic components of biomembranes, leading to the change in the permeability of biomembranes and permitting heavy metals to enter easily into microbial cells (Shen et al., 2006).Furthermore, this rupture in the cell membrane/wall of the fungus is correlated with TEM-EDAX result **Fig.4 (b) II** where interrupted dark electron region could be observed throughout the cell membrane of the fungal cell.

The total bioaccumulated multimetal quantities per unit mass of biomass on dry weight basis (specific uptake capacity) represented by q_m reduced in case of MML (3.40 mg/g) when compared to MM (6.70 mg/g) as shown in **(Table.1.)**.

Earlier we have found that the heavy metal bioaccumulation potential of fungus varies from species to species, on the concentration of heavy metals and also on the dynamics of the cocktail of heavy metals (Dey et al., 2016).Thus in this study the uptake of heavy metals by *A.fumigatus* was affected with the introduction of the co-contaminant lindane.

Furthermore, it is noteworthy that biodegradation of lindane by microorganisms have been studied by several researchers (Ceci et al., 2015; Garg et al., 2012; Lodha et al., 2007; Mougin et al., 1996; Sagar and Singh, 2011).Yet, the degradation of the pesticide lindane in the presence of hexametal by a fungus has not been studied so far.

4.2. Physiological analysis of metal accumulation by *A.fumigatus*

4.2.1. Scanning Electron Microscope (SEM) analysis

Scanning Electron Microscope (SEM) analysis provided the mapping of surface details. The metal resistant microorganisms exhibit various structural and morphological adaptations under multimetal and organic contaminant stress. The SEM micrographs showed a clear difference between the control **Fig.3 (a)** and the mycelia stressed with 30 mg/L multimetal and 30 mg/L multimetal + 30 mg/L lindane **Fig.3 (b-c)**. The fungal hyphae in control is loosely packed and ribbon like in the multimetal free and lindane free biomass **Fig.3 (a).**The mycelia in the presence of 30 mg/L multimetal brought slight shrinkage **Fig.3 (b)**.Lundy et al.,(2001) observed decrease in the mycelial length of the fungus *Achlya bisexualis* in the presence of the metals Cu, Co, Hg, Zn and Cd. Toxic metals bind and chelate with the cell membrane and consequently result in the distortion of the cell structure as well as loss of cell function (De Silóniz et al., 2002;Yilmazer and Saracoglu, 2009).

Thus in a stressful environment of the individual metals, the mycelia aggregates and reduces the exposed surface area in order to facilitate the concentration of intracellular polysaccharides, metal chelating pigments and agents like melanin and siderophores resulting in the development of pressure within the fungal mycelia which accounts for the chelation of metals (Gadd, 2007).

Interestingly, the impact of 30 mg/L multimetal + 30 mg/L lindane on the mycelial structure had pronounced rough surface when compared to the 30 mg/L multimetal. Furthermore, from **Fig.3 (c)** granular deposition of the pesticide lindane on the mycelia can be observed, which indicates the adsorption of lindane on the surface of the fungal strain. This observation is substantiated with the research (Ghosh et al., 2009) who postulated the involvement of the hydrophobic interaction of the lipid component in the fungal biomass for the adsorption of lindane onto its biomass. Furthermore, the adsorption of lindane onto the fungal biomass surface corroborates the reduced chelation of the metals Cd, Cr, Cu and Ni in this study.

4.2.2. Transmission Electron Microscopy coupled with EDX analysis

Transmission Electron Microscopy coupled with EDX technique detailed the intracellular structures in the fungal cell. Thus, it demonstrates the mechanisms of living processes at physiological level. The metal resistant microorganisms exhibit preferences for metal uptake in a multimetal and organic contaminant stress. The TEM-EDX micrographs depicted, the fungal isolate accumulated Ni and Zn throughout the cell membrane and cytoplasm while Cd, Cr, Cu and Pb in the cell membrane/cell wall in case of 30 mg/L initial multimetal **Fig.4 (a) (I-IX)**. There is an observance of dark electron region bordering throughout the fungal cell **Fig (a)II.**

When the fungal biomass was exposed to the 30 mg/L multimetal + 30 mg/L lindane **Fig.4 (b) (I-IX)**, this strain had the tendency to accumulate the metals Cd, Cu and Pb in its cell wall/membrane while the metals Cr, Ni and Zn in its cell membrane and cytoplasm as

depicted in the figures. So, the metals Cr, Ni and Zn had the tendency to be bioaccumulated onto the fungal cell cytoplasm including the intracellular vacuoles in both the exposures. These intracellular vacuoles hoard the thiol containing compounds such as GSH and metallothioneins which are responsible in channelizing the metals into the vacuoles to reduce the metal load in the cytoplasm (Ge et.al., 2011).Furthermore from the micrograph **Fig.4 (b) VII** it can be inferred that a distinct dense layer of the Pb being formed onto the cell membrane which denotes the higher accumulation of this metal due to the rupture of the cell wall in the presence of the pesticide lindane**.**

Srivastava and Thakur, (2006) studied the metal rich area of the cell wall by TEM-EDX, confirming the role of cell wall of the fungal strain *Aspergillus niger* as the primary accumulator of chromate from the solution. Thus chitin and chitosan components of the fungal cell wall are instrumental in sequestration of metal ions (De Lima et al., 2013; Purchase et al., 2009).

4.2.3. Fourier Transform Infrared Spectroscopy (FTIR) analysis

As biosorption have significant roles in metal remediation, it is pertinent to know the various functional groups that aid in binding of metals in the fungal biomass. The FTIR spectra of the lyophilized fungal biomass of *A.fumigatus* after metal uptake were examined for the masking and presence of the functional groups involved in the metal chelation.

The dominant functional groups present in the biotic control are O-H and N-H stretching, C-H stretching, C=O stretching in carboxyl or amide I band groups, N-H bending in amide III and C-N stretching in –CO-NH- groups, C-OH and C-O-P stretching, nitro and disulphide groups. So, when compared with the biotic control **(Fig.5)**, in case of the 30 mg/L multimetal laden biomass there were masking of the C-H stretching group. Guibal et al.,(1995) studied amides in metal chelating in *Aspergillus, Penicillum, Mucor*. The NH_2 group is a chemical active site in the chitin of the fungal cell wall responsible for metal binding (Niu and

514 Volesky, 2006). Further, chitin, glucan, mannan and proteins are the components of fungal
515 cell walls. Apart from these it also contains other polysaccharides, lipids and pigments
516 (melanin) which facilitate binding of many metal ions. This metabolism independent process
517 comprising of the negatively charged groups of carboxyl, phenolic, hydroxyl, carbonyl and
518 methoxyl are important as they bind to the oxygen binding sites which are present in the
519 phenolic polymers and melanins of the fungal cell wall (Gadd, 2004).These functional groups
520 of the cell wall moderate the accumulation of the toxic metals inside the cytoplasm that
521 houses most of the enzymes which are required for cell metabolism (Ge et.al., 2011).
522 In case of 30 mg/L multimetal + 30 mg/L lindane laden biomass there was masking of the
523 carboxyl/amide group stretching. Thus, confirming the adsorption of the pesticide lindane
524 with the low molecular weight organic acids viz. carboxylic and amino groups present in the
525 cell wall of the fungus. This in turn may lead to competition with the binding of the inorganic
526 heavy metals onto the fungal cell. Thus, enhancing the bioavailability and mobility of the
527 pesticide for degradation by the microbe (van de Kreeke et al., 2010). This hypothesis also
528 validates with SEM micrograph **Fig.3 (c)** which depicts adsorption of lindane on the fungal
529 biomass resulting in the interference of the metal uptake.
530 Thus, overall this fungus adopts stratagems to alleviate multimetal and pesticide which
531 include adsorption of lindane pesticide on the fungal biomass. Intracellular metal import to
532 the cytoplasm and chelation of metals to the fungal cell wall components. These mechanisms
533 work in a coordinated manner to maintain homoeostasis in the fungus.

534 **5.Conclusions**

535 This study investigated the remediation of pesticide lindane and biosorption and
536 bioaccumulation of multimetal (mixture of cadmium, chromium, copper, nickel, lead, zinc)
537 by newly isolated *A.fumigatus* PD-18 from composite medium. The results deduced the
538 enhanced uptake of metals Pb and Zn in fungus in presence of lindane. Remarkably, lindane

was degraded below permitted limit value as prescribed by European Community legislation. However, the bioaccumulation of metals Cd, total Cr, Cu, Ni ions was impeded on addition of lindane.

Lindane adsorption on the fungal biomass is clearly depicted by SEM analysis. The localization of the metals Cr, Ni and Zn in the cytoplasm was revealed by TEM-EDX while FTIR analysis shed light on the competition between metal sequestration and the adsorption of pesticide lindane on the functional groups of carboxyl/amide.

Overall this study provides the invaluable detailed information that *A.fumigatus* adopts the strategy of removal of metals from different complex contaminant conditions. Essentially the extracellular metal chelation onto fungal cell surface and the intracellular binding of metals into the organelles in the cytoplasm. Thus, this knowledge may lead to the optimization of recovery process of the metals from the biomass and development of a field-worthy remediation systems for complex mixtures.

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7. Conflict of interest statement

The authors declare that they have no conflict of interest.

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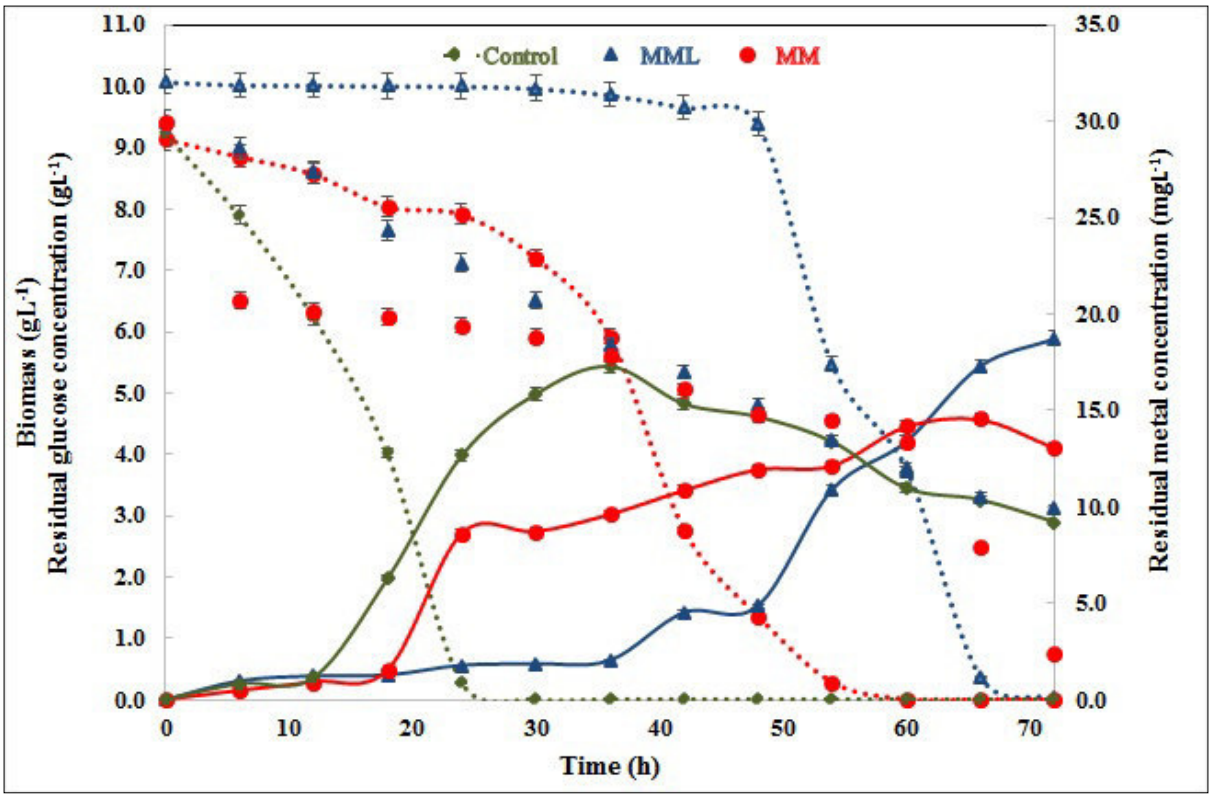

Fig.1: Residual glucose concentration (dotted lines), residual metal concentration (symbols) in broth and biomass production (solid lines) by *A. fumigatus* in presence of 1% glucose in BC; 30 mg L^{-1} MM; 30 mg L^{-1} MM & 30 mg L^{-1} lindane

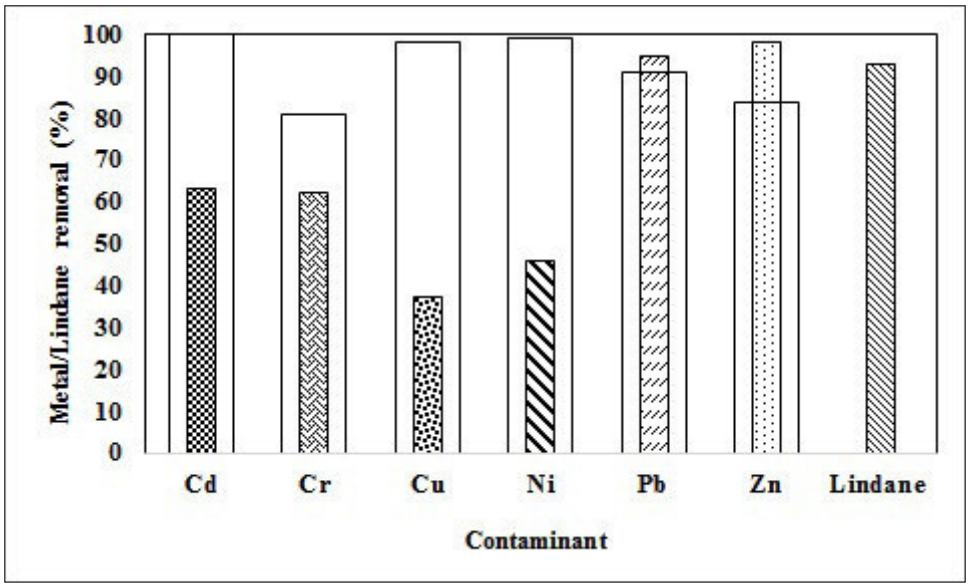

Fig.2: Removal of metals in presence of 1% glucose, 30 mg L^{-1} multimetal and absence (blank bars) or presence (patterned bar) of 30 mg L^{-1} lindane

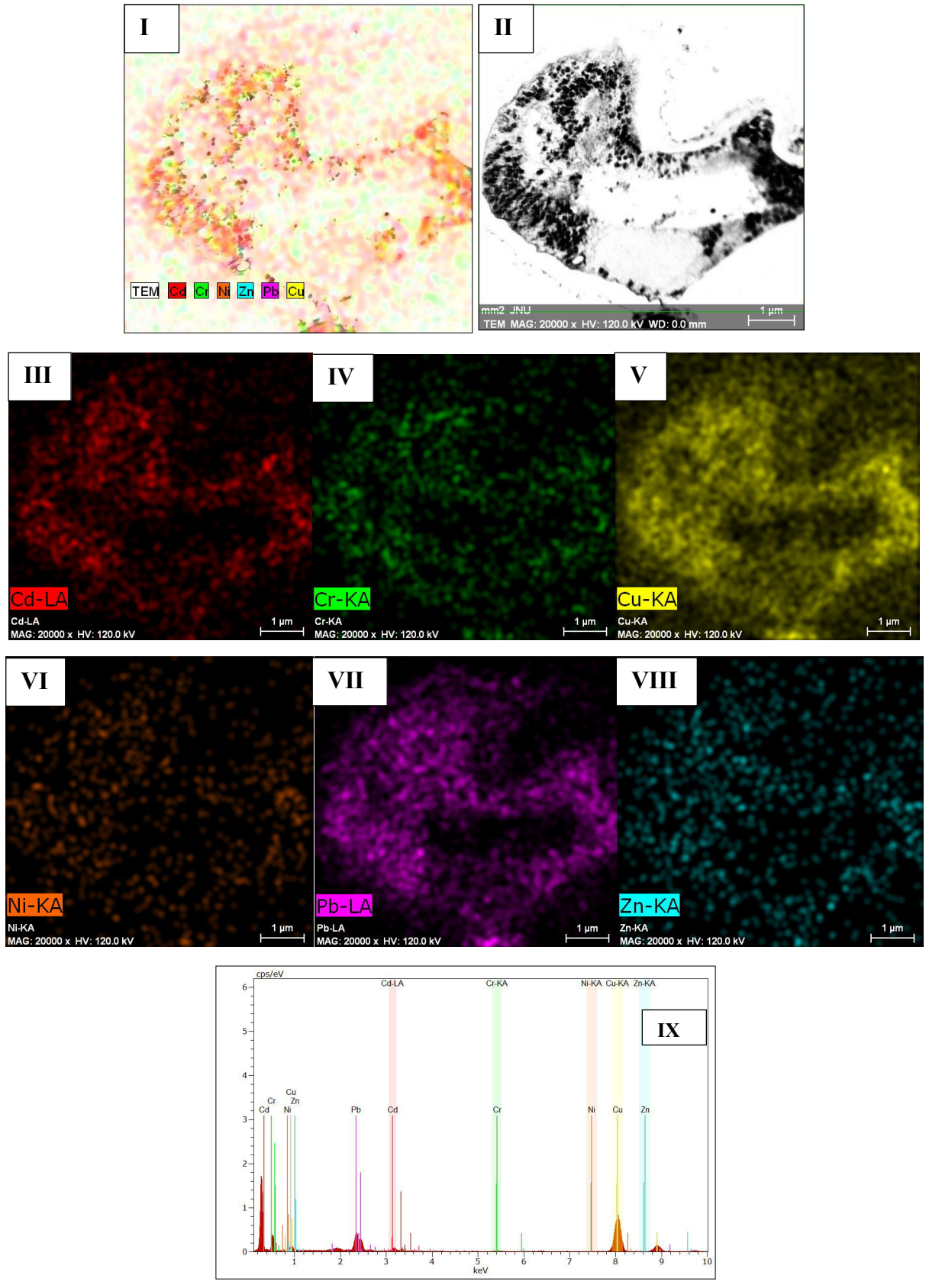

Fig.3 (a): TEM-EDAX mapping of pellets of *A. fumigatus* in 30 mg L^{-1} multimetal.
(II) TEM micrograph in presence of multimetal (Cd, Cr, Cu, Ni, Pb, Zn); (I-IX) EDAX depicting the exact location of accumulation of individual metal onto the different organelles of the fungal cell; (IX) EDAX graph confirm the presence of individual metals inside the fungal cell

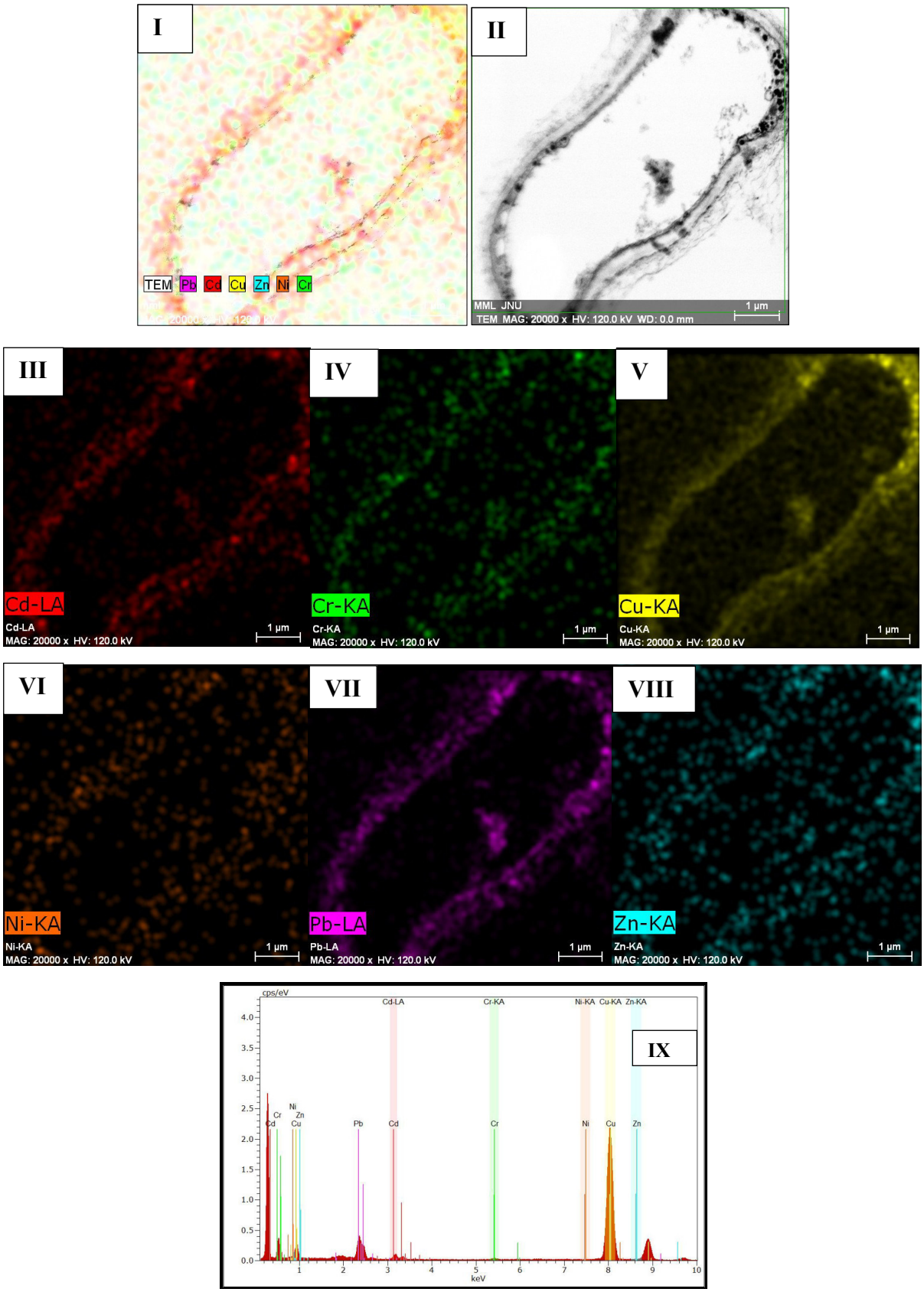

Fig.3 (b): TEM-EDAX mapping of pellets of *A. fumigatus* in 30 mg L^{-1} multimetal and 30 mg L^{-1} lindane. (II) TEM micrograph in presence of multimetal (Cd, Cr, Cu, Ni, Pb, Zn); (I-IX) EDAX depicting the exact location of accumulation of individual metal onto the different organelles of the fungal cell; (IX) EDAX graph confirm the presence of individual metals inside the fungal cell

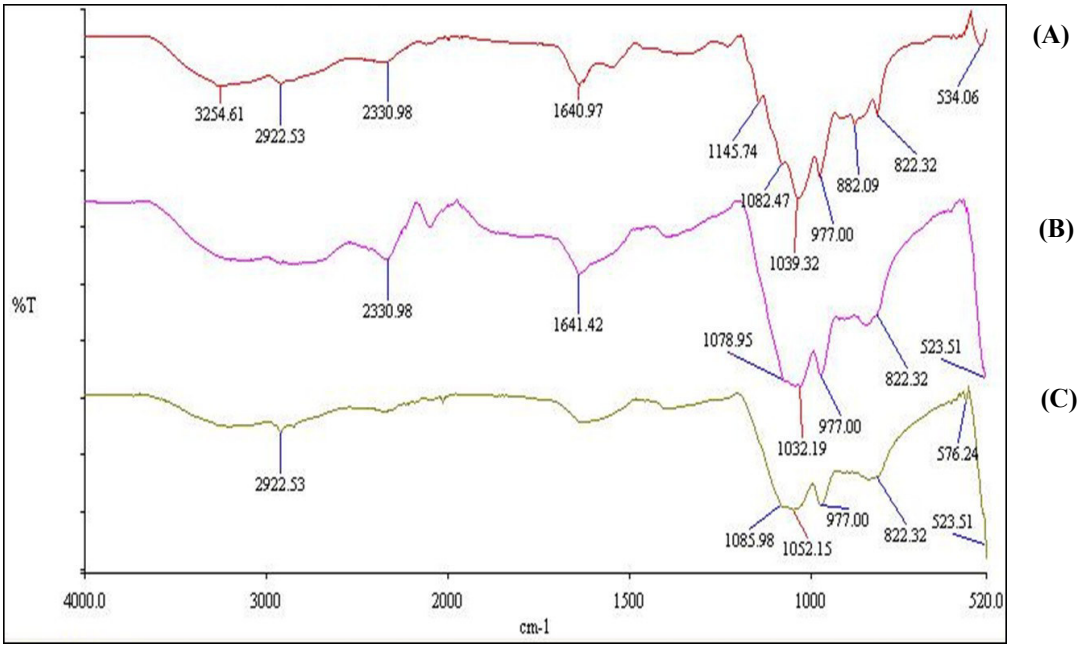

Fig.4: FTIR spectra of pellets of *A. fumigatus* (A) In absence of multimetal and lindane (Biotic control); (B) 30 mg L^{-1} multimetal (MM) (C) 30 mg L^{-1} multimetal + 30 mg L^{-1} lindane (MML)

Declaration of interests

 The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

The contributions of the authors have been acknowledged

Anushree Malik and Priyadarshini Dey: Conceptualization, methodology, validation, formal analysis, investigation, resources, data curation, Writing-Original draft preparation

Abhishek Mishra, Dileep Kumar Singh, Martin von Bergen, Nico Jehmlich: Writing-Review and Editing

Anushree Malik: Supervision

Anushree Malik: Funding acquisition

Figure Legends

Figure 1: Residual glucose concentration (dotted lines), residual metal concentration (symbols) in broth and biomass production (solid lines) by *A.fumigatus* in presence of 1% glucose in BC; 30 mg L^{-1} MM; 30 mg L^{-1} MM & 30 mg L^{-1} lindane

Figure 2: Removal of metals in presence of 1% glucose, 30 mg L^{-1} multimetal and absence (blank bars) or presence (patterned bar) of 30 mg L^{-1} lindane

Figure 3 (a): TEM-EDAX mapping of pellets of *A. fumigatus* in 30 mg L^{-1} multimetal.
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Figure 3 (b): TEM-EDAX mapping of pellets of *A. fumigatus* in 30 mg L^{-1} multimetal and 30 mg L^{-1} lindane.
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Figure 4: FTIR spectra of pellets of *A. fumigatus* (A) In absence of multimetal and lindane (Biotic control); (B) 30 mg L^{-1} multimetal (MM) (C) 30 mg L^{-1} multimetal + 30 mg L^{-1} lindane (MML)

Supplementary Fig.S1: Biomass production, residual glucose concentration, residual multimetal concentration, residual lindane concentration during growth of *A.fumigatus* in the presence of 1% glucose, 30 mg L^{-1} multimetal and 30 mg L^{-1} lindane

Supplementary Fig. S2: Scanning Electron micrographs of pellets of *A. fumigatus* (a) in absence of multimetal and lindane (Biotic control); (b) in 30 mg L^{-1} multimetal (MM); (c) in 30 mg L^{-1} multimetal + 30 mg L^{-1} lindane (MML)

Supplementary Fig. S3(a): Chromatogram of the organochlorine compounds detected during the degradation of lindane with *A.fumigatus* in the presence of 1% glucose, 30 mg L^{-1} multimetal and 30 mg L^{-1} lindane at 0 h

Supplementary Fig. S3(b): Chromatogram of the organochlorine compounds detected during the degradation of lindane with *A.fumigatus* in the presence of 1% glucose, 30 mg L^{-1} multimetal and 30 mg L^{-1} lindane at 72 h

Supplementary Fig.S4: Biomass production during growth of *A.fumigatus* in the presence of 1% glucose, 30 mg L^{-1} multimetal and 30 mg L^{-1} lindane

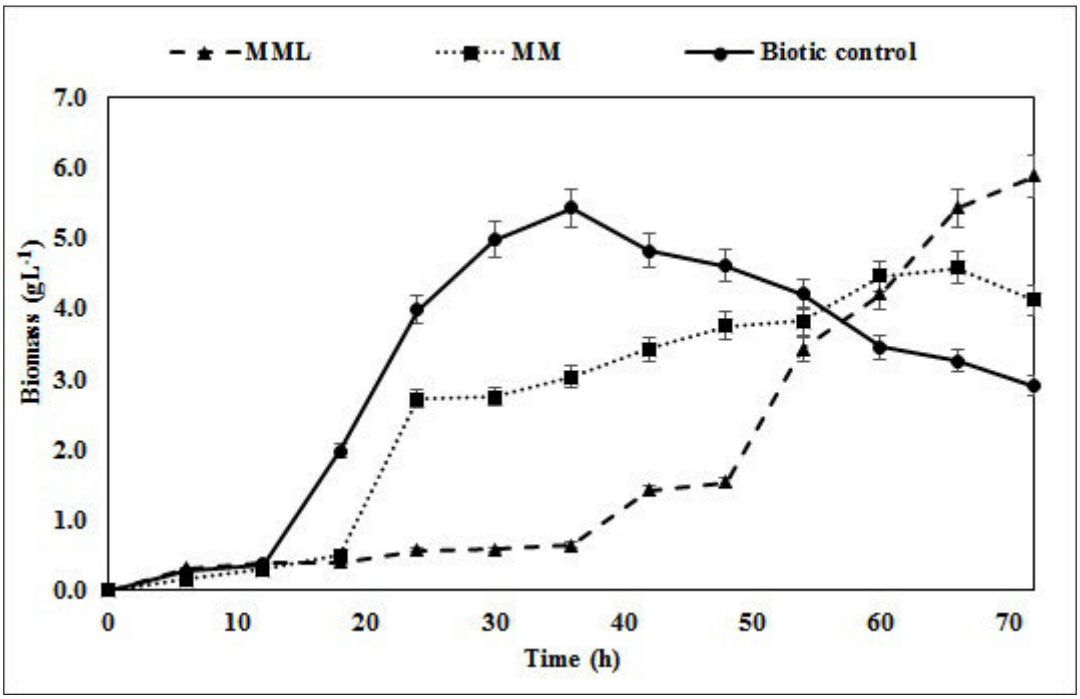
Supplementary Fig.S5: Residual glucose concentration during growth of *A.fumigatus* in the presence of 1% glucose, 30 mg L^{-1} multimetal and 30 mg L^{-1} lindane

Supplementary Fig.S6: Residual multimetal concentration during growth of *A.fumigatus* in the presence of 1% glucose, 30 mg L^{-1} multimetal and 30 mg L^{-1} lindane

Table Legends

Supplementary Table.1. Summary of *A.fumigatus* performance in terms of cube root growth kinetic constant, biomass and multimetal accumulation under the exposures of Multimetal and Multimetal + Lindane

Supplementary Table.2. One-way MANOVA results showing the effect of lindane addition on the multimetal uptake by *A.fumigatus* (significant at $p<0.05$)



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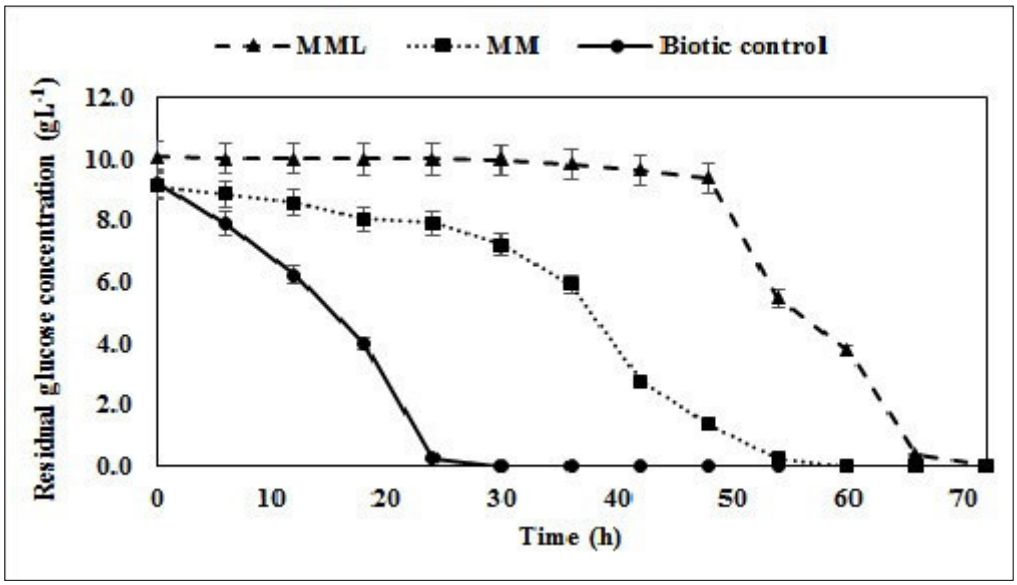

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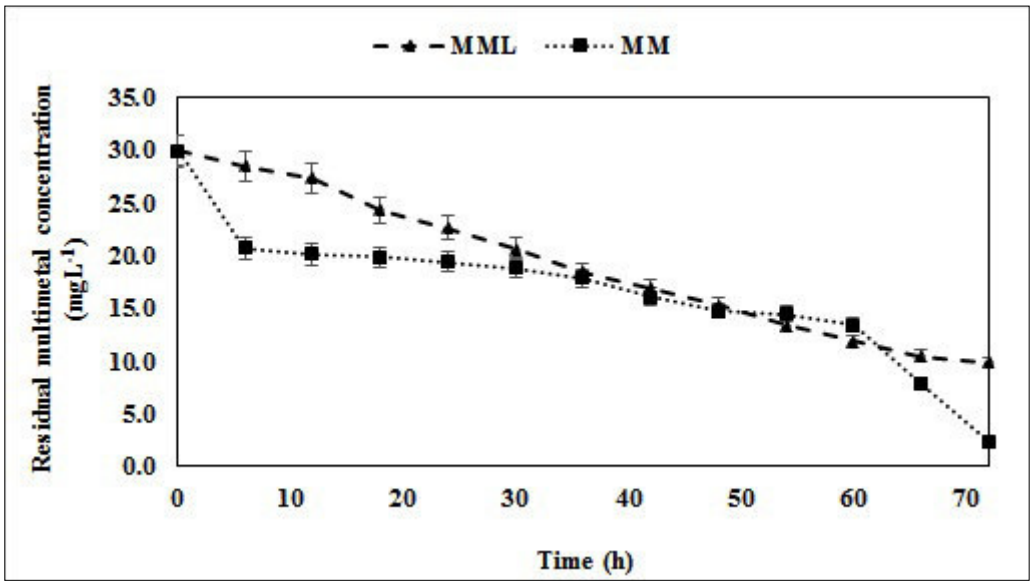

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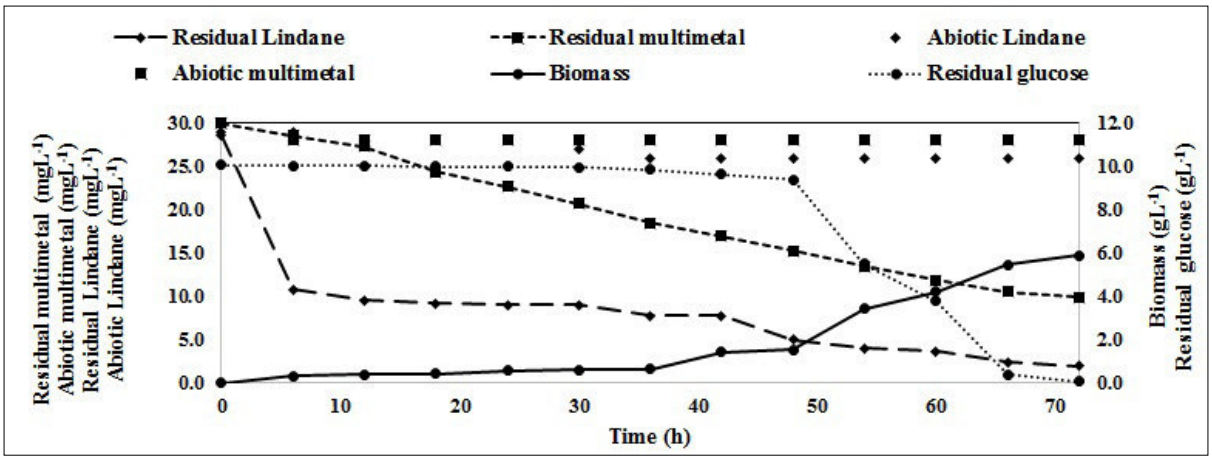

Supplementary Fig.S4: Biomass production, residual glucose concentration, residual multimetal concentration, residual lindane concentration during growth of *A.fumigatus* in the presence of 1% glucose, 30 mg L^{-1} multimetal and 30 mg L^{-1} lindane

Area % Report

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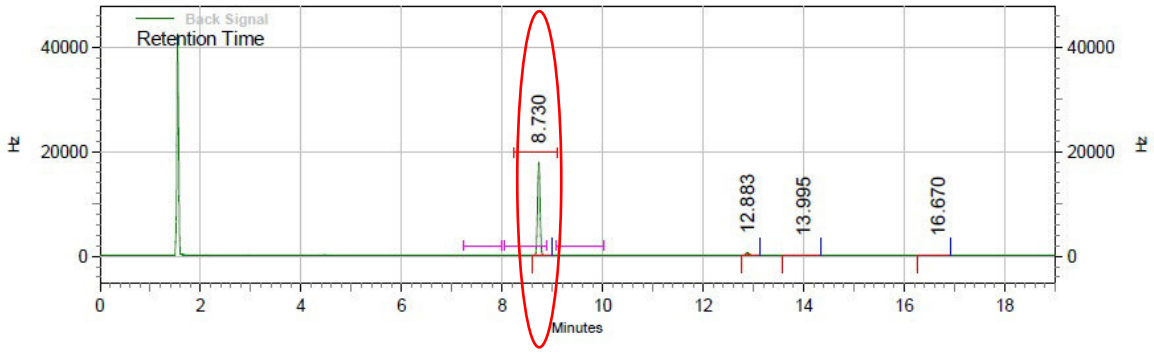

Back Signal
Results

Retention Time	Area	Area %	Height	Height %
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12.883	18900906	3.73	3898040	2.76
13.995	5583057	1.10	342833	0.24
16.670	1938565	0.38	101857	0.07
Totals	506081199	100.00	141182840	100.00

Supplementary Fig. S5(a): Chromatogram of the organochlorine compounds detected during the degradation of lindane with *A.fumigatus* in the presence of 1% glucose, 30 mg L^{-1} multimetal and 30 mg L^{-1} lindane at 0 h

Area % Report

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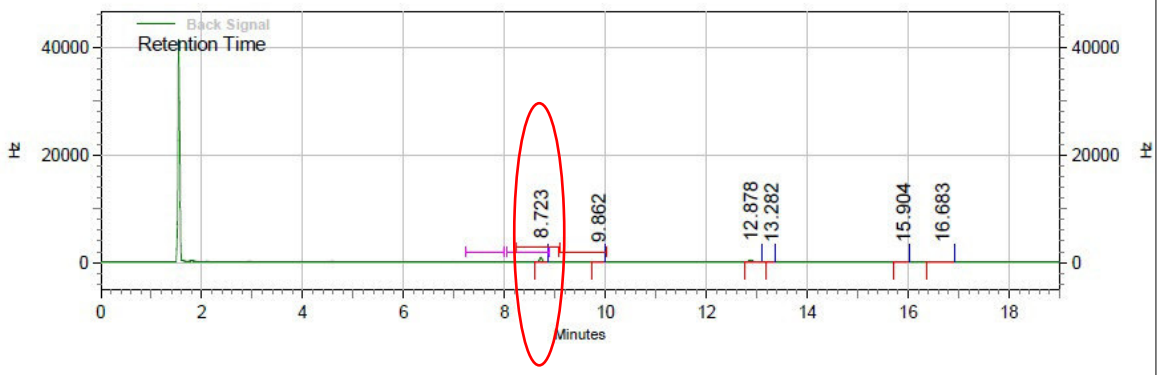
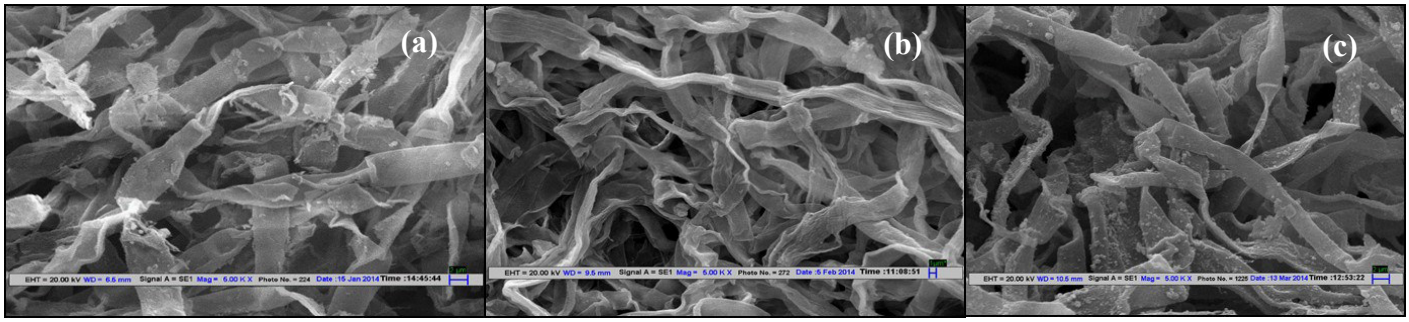


Back Signal
Results

Retention Time	Area	Area %	Height	Height %
8.723	20835456	60.18	6236452	72.48
9.862	1489342	4.30	231093	2.69
12.878	8758601	25.30	1660240	19.29
13.282	1329379	3.84	235763	2.74
15.904	1056877	3.05	165127	1.92
16.683	1155073	3.34	76279	0.89
Totals	34624728	100.00	8604954	100.00

Supplementary Fig. S5(b): Chromatogram of the organochlorine compounds detected during the degradation of lindane with *A.fumigatus* in the presence of 1% glucose, 30 mg L^{-1} multimetal and 30 mg L^{-1} lindane at 72 h



Supplementary Fig.S6: Scanning Electron micrographs of pellets of *A. fumigatus* (a) in absence of multimetal and lindane (Biotic control); (b) in 30 mg L^{-1} multimetal (MM); (c) in 30 mg L^{-1} multimetal + 30 mg L^{-1} lindane (MML)

Supplementary Table.1. Summary of *A.fumigatus* performance in terms of cube root growth kinetic constant, biomass and multimetal accumulation under the exposures of Multimetal and Multimetal + Lindane

Type of contaminants	**Cube root growth kinetic constant k** ($g^{1/3} L^{-1/3} h^{-1}$)	**Maximum biomass obtained** ($g L^{-1}$)	**Multimetal accumulation** ($mg L^{-1}$)	**Specific uptake capacity qm** ($mg g^{-1}$)	**Time taken for glucose consumption (h)**	**Metal removal efficiency (%)**
Biotic Control	0.0562	5.42 ± 0.01	-	-	36	-
Multimetal	0.0226	4.59 ± 0.01	27.59 ± 0.09	6.7	66	91
Multimetal+Lindane	0.0211	5.89 ± 0.03	20.04 ± 0.02	3.4	~72	67

Supplementary Table.2. One-way MANOVA results showing the effect of lindane addition on the multimetal uptake by *A.fumigatus* (significant at p<0.05)

Metals	F-test values	p values
Cd	47.2	< 0.001
Cr	6.0	0.022
Cu	185.0	< 0.001
Ni	326.3	< 0.001
Pb	5.5	0.028
Zn	1693.6	< 0.001